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(54) Title: RIP: NOVEL HUMAN PROTEIN INVOLVED IN TUMOR NECROSIS FACTOR SIGNAL TRANSDUCTION, AND SCREENING ASSAYS

(57) Abstract

The invention relates to a human Receptor Interacting Protein (hRIP), nucleic acids which encode hRIP and methods of using the subject compositions; in particular, methods such as hRIP-based in vitro binding assays and phosphorylation assays for screening chemical libraries for lead compounds for pharmacological agents.

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*RIP:Novel Human Protein Involved in Tumor Necrosis Factor Signal Transduction, and Screening Assays*

## INTRODUCTION

Field of the Invention

The field of this invention is a novel human kinase involved in tumor necrosis factor signal transduction and its use in drug screening.

Background

Tumor necrosis factor (TNF) is an important cytokine involved in the signaling of a number of cellular responses including cytotoxicity, anti-viral activity, immun.-regulatory activities and the transcriptional regulation of a number of genes. The TNF receptors (TNF-R1 and TNF-R2) are members of the larger TNF receptor superfamily which also includes the Fas antigen, CD27, CD30, CD40, and the low affinity nerve growth factor receptor. Members of this family have been shown to participate in a variety of biological properties, including programmed cell death, antiviral activity and activation of the transcription factor NF-κB in a wide variety of cell types.

Accordingly, it is desired to identify agents which specifically modulate transduction of TNF receptor family signalling. Unfortunately, the components of the signalling pathway remain largely unknown; hence, the reagents necessary for the development of high-throughput screening assays for such therapeutics are unavailable. Elucidation of TNF receptor family signal transduction pathways leading to NF-κB activation would provide valuable insight into mechanisms to alleviate inflammation. In particular, components of this pathway would provide valuable targets for automated, cost-effective, high throughput drug screening and hence would have immediate application in a broad range of domestic and international pharmaceutical and biotechnology drug development programs.

Relevant Literature

Stanger et al. (1995) Cell 81, 513-523 report the existence of a Receptor Interacting Protein (RIP) and its functional expression. VanArsdale and Ware (1994) J Immunology 153:3043-3050 describe proteins associated with TNF-R1. The cloning and amino acid sequencing of TNF-R1 is disclosed in Schall et al (1990) Cell 61, 361 and Loetscher et al (1990) Cell 61, 351; the identification of a "death domain" in TNF-R1 is disclosed in Tartaglia et al. (1993) Cell 74:845-853. The cloning and amino acid sequence of a TNF-R associated death domain protein (TRADD) is described by Hsu et al. (1995) Cell 81, 495-504. The cloning and amino acid sequence of the Fas antigen is disclosed in Itoh et al (1991)

Cell 66, 233-243. For a recent review, see Smith et al. (1994) Cell 76:959-962 and Vandenabelle et al. (1995) Trends Cell Biol. 5, 392-399.

## SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to a human Receptor Interacting Protein (hRIP). The compositions include nucleic acids which encode hRIP, hRIP kinase domains, and recombinant proteins made from these nucleic acids. The invention also provides methods for screening chemical libraries for lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated hRIP activity or hRIP-dependent signal transduction. In one embodiment, the methods involve incubating a mixture of hRIP, a natural intracellular hRIP substrate or binding target and a candidate pharmacological agent and determining if the presence of the agent modulates the ability of hRIP to selectively phosphorylate the substrate or bind the binding target. Specific agents provide lead compounds for pharmacological agents capable of disrupting hRIP function.

## DETAILED DESCRIPTION OF THE INVENTION

A human RIP-encoding nucleic acid sequence is set out in SEQ ID NO: 1. A human RIP kinase domain-encoding nucleic acid sequence is set out in SEQ ID NO: 1, nucleotides 1-900. A human RIP amino acid sequence is set out in SEQ ID NO: 2; and a hRIP kinase domain sequence is set out in SEQ ID NO:2, residues 1-300.

Natural nucleic acids encoding hRIP are readily isolated from cDNA libraries with PCR primers and hybridization probes containing portions of the nucleic acid sequence of SEQ ID NO:1. For example, we used low stringency hybridization at 42°C (hybridization buffer: 20% formamide, 10 % Denhardt, 0.5% SDS, 5X SSPE; with membrane washes at room temperature with 5X SSPE/0.5% SDS) with a 120 base oligonucleotide probe (SEQ ID NO: 1, nucleotides 1728-1847) to isolate a native human RIP cDNA from a library prepared from human umbilical vein endothelial cells. In addition, synthetic hRIP-encoding nucleic acids may be generated by automated synthesis.

The subject nucleic acids are recombinant, meaning they comprise a sequence joined to a nucleotide other than that to which sequence is naturally joined and isolated from a natural environment. The nucleic acids may be part of hRIP-expression vectors and may be incorporated into cells for expression and screening, transgenic animals for functional studies

(e.g. the efficacy of candidate drugs for disease associated with expression of a hRIP), etc. These nucleic acids find a wide variety of applications including use as templates for transcription, hybridization probes, PCR primers, therapeutic nucleic acids, etc.; use in detecting the presence of hRIP genes and gene transcripts, in detecting or amplifying nucleic acids encoding additional hRIP homologs and structural analogs, and in gene therapy applications.

The invention provides efficient methods of identifying pharmacological agents or lead compounds for agents active at the level of a hRIP modulatable cellular function, particularly hRIP mediated TNF receptor or Tumor necrosis factor receptor associated Factor -2 (TRAF2) or TRADD-induced signal transduction. For example, we have found that a binding complex comprising TNF R1, TRADD, and hRIP exists in TNF-stimulated cells. Generally, the screening methods involve assaying for compounds which interfere with a hRIP activity such as kinase activity or TRAF2 or TRADD binding. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in in vitro and in vivo assays to optimize activity and minimize toxicity for pharmaceutical development. Target therapeutic indications are limited only in that the target cellular function be subject to modulation, usually inhibition, by disruption of the formation of a complex comprising hRIP and one or more natural hRIP intracellular binding targets including substrates or otherwise modulating hRIP kinase activity. Target indications may include infection, genetic disease, cell growth and regulatory or immunolgic dysfunction, such as neoplasia, inflammation, hypersensitivity, etc.

A wide variety of assays for binding agents are provided including labeled in vitro kinase assays, protein-protein binding assays, immunoassays, cell based assays, etc. The hRIP compositions used in the methods are recombinantly produced from nucleic acids having the disclosed hRIP nucleotide sequences. The hRIP may be part of a fusion product with another peptide or polypeptide, e.g. a polypeptide that is capable of providing or enhancing protein-protein binding, stability under assay conditions (e.g. a tag for detection or anchoring), etc.

The assay mixtures comprise one or more natural intracellular hRIP binding targets including substrates, such as TRADD, TRAF2, or, in the case of an autophosphorylation

assay, the hRIP itself can function as the binding target. In one embodiment, the mixture comprises a complex of hRIP, TRADD and TNFR1. A hRIP derived pseudosubstrate may be used or modified (e.g. A to S/T substitutions) to generate effective substrates for use in the subject kinase assays as can synthetic peptides or other protein substrates. Generally, hRIP-specificity of the binding agent is shown by kinase activity (i.e. the agent demonstrates activity of an hRIP substrate, agonist, antagonist, etc.) or binding equilibrium constants (usually at least about $10^6 M^{-1}$, preferably at least about $10^8 M^{-1}$, more preferably at least about $10^9 M^{-1}$). A wide variety of cell-based and cell-free assays may be used to demonstrate hRIP-specific binding; preferred are rapid in vitro, cell-free assays such as mediating or inhibiting hRIP-protein (e.g. hRIP-TRADD) binding, phosphorylation assays, immunoassays, etc.

The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc. which may be used to facilitate optimal binding and/or reduce non-specific or background interactions, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

In a preferred in vitro, binding assay, a mixture of at least the kinase domain of hRIP, one or more binding targets or substrates and the candidate agent is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the hRIP specifically binds the cellular binding target at a first binding affinity or phosphoylates the substrate at a first rate. After incubation, a second binding affinity or rate is detected. Detection may be effected in any convenient way. For cell-free binding assays, one of the components usually comprises or is coupled to a label. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components. For example, the label may be detected bound to the solid substrate or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

1. Protocol for hRIP autophosphorylation assay.

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.

- hRIP: $10^{-8}$ - $10^{-5}$ M biotinylated hRIP kinase domain, residues 1-300 at 20 µg/ml in PBS.

- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.

- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

-[ $^{32}$P]γ-ATP 10x stock: 2 x $10^{-5}$ M cold ATP with 100 µCi [ $^{32}$P]γ-ATP. Place in the 4°C microfridge during screening.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock Neutralite avidin per well overnight at 4°C.
- Wash 2 times with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2 times with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 40 µl biotinylated hRIP (0.1-10 pmoles/40 ul in assay buffer)
- Add 10 µl compound or extract.
- Add 10 µl [$^{32}$P]γ-ATP 10x stock.
- Shake at 30°C for 15 minutes.
- Incubate additional 45 minutes at 30°C.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.

- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no RIP added)

b. cold ATP to achieve 80% inhibition.

2. Protocol for hRIP - substrate phosphorylation assay.

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.

- hRIP: $10^{-8}$ - $10^{-5}$ M hRIP at 20 µg/ml in PBS.

- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.

- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM BME, 1 mg/ml BSA, cocktail of protease inhibitors.

-[ $^{32}$P]γ-ATP 10x stock: 2 x $10^{-5}$ M cold ATP with 100 µCi [ $^{32}$P]γ-ATP. Place in the 4°C microfridge during screening.

- Substrate: 2 x $10^{-6}$ M biotinylated synthetic peptide kinase substrate at 20 µg/ml in PBS.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock Neutralite avidin per well overnight at 4°C.

- Wash 2 times with 200 µl PBS.

- Block with 150 µl of blocking buffer.

- Wash 2 times with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.

- Add 40 µl hRIP (0.1-10 pmoles/40 ul in assay buffer)

- Add 10 µl compound or extract.

- Shake at 30°C for 15 minutes.

- Add 10 µl [$^{32}$P]γ-ATP 10x stock.

- Add 10 µl substrate.

- Shake at 30°C for 15 minutes.
- Incubate additional 45 minutes at 30°C.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no RIP added)

b. cold ATP to achieve 80% inhibition.

3. Protocol for hRIP - TRADD binding assay.

A. Reagents:

- Anti-myc antibody: 20 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM β-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.
- $^{33}$P hRIP 10x stock: $10^{-8}$ - $10^{-6}$ M "cold" hRIP (full length) supplemented with 200,000-250,000 cpm of labeled hRIP (HMK-tagged) (Beckman counter). Place in the 4°C microfridge during screening.
- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml PBS.
- TRADD: $10^{-8}$ - $10^{-5}$ M myc eptitope-tagged TRADD in PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock anti-myc antibody per well overnight at 4°C.
- Wash 2X with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2X with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 10 µl compound or extract.
- Add 10 µl $^{33}$P-RIP (20,000-25,000 cpm/0.1-10 pmoles/well = $10^{-9}$ - $10^{-7}$ M final

concentration).

- Shake at 25°C for 15 minutes.
- Incubate additional 45 minutes at 25°C.
- Add 40 µl eptitope-tagged TRADD (0.1-10 pmoles/40 ul in assay buffer)
- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no hRIP added)

b. Soluble (non-tagged TRADD) to achieve 80% inhibition.

4. Protocol for hRIP - TRAF2 binding assay.

A. Reagents:

- Anti-myc antibody: 20 µg/ml in PBS.
- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.
- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM β-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.
- $^{33}$P hRIP 10x stock: $10^{-8}$ - $10^{-6}$ M "cold" hRIP kinase domain, residues 1-300, supplemented with 200,000-250,000 cpm of labeled hRIP kinase domain (HMK-tagged) (Beckman counter). Place in the 4°C microfridge during screening.
- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml PBS.
- TRAF2: $10^{-8}$ - $10^{-5}$ M myc eptitope-tagged TRAF2 in PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock anti-myc antibody per well overnight at 4°C.
- Wash 2X with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2X with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 10 µl compound or extract.
- Add 10 µl $^{33}$P-RIP kinase domain (20,000-25,000 cpm/0.1-10 pmoles/well =$10^{-9}$- $10^{-7}$ M final concentration).
- Shake at 25°C for 15 minutes.
- Incubate additional 45 minutes at 25°C.
- Add 40 µl eptitope-tagged TRAF2 (0.1-10 pmoles/40 ul in assay buffer)
- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding (no hRIP kinase domain added)

b. Soluble (non-tagged TRAF2) to achieve 80% inhibition.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: RIP: NOVEL HUMAN PROTEIN INVOLVED IN TUMOR NECROSIS FACTOR SIGNAL TRANSDUCTION, AND SCREENING ASSAYS

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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(C) CLASSIFICATION:

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(C) REFERENCE/DOCKET NUMBER: T95-006/PCT

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2016 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2013

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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        355                 360                 365
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    370                 375                 380
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His Leu Tyr Gly Ser Arg Met Asp Arg Gln Thr Lys Gln Gln Pro Arg
385                 390                 395                 400
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CAT GAC CCT TTT GCA CAG CAA AGA CCT TAC GAG AAT TTT CAG AAT ACA      1296
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Glu Gly Lys Gly Thr Val Tyr Ser Ser Ala Ala Ser His Gly Asn Ala
        435                 440                 445
GTG CAC CAG CCC TCA GGG CTC ACC AGC CAA CCT CAA GTA CTG TAT CAG      1392
Val His Gln Pro Ser Gly Leu Thr Ser Gln Pro Gln Val Leu Tyr Gln
    450                 455                 460
AAC AAT GGA TTA TAT AGC TCA CAT GGC TTT GGA ACA AGA CCA CTG GAT      1440
Asn Asn Gly Leu Tyr Ser Ser His Gly Phe Gly Thr Arg Pro Leu Asp
465                 470                 475                 480
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CCA GGA ACA GCA GGT CCC AGA GTT TGG TAC AGG CCA ATT CCA AGT CAT      1488
Pro Gly Thr Ala Gly Pro Arg Val Trp Tyr Arg Pro Ile Pro Ser His
                485                 490                 495
ATG CCT AGT CTG CAT AAT ATC CCA GTG CCT GAG ACC AAC TAT CTA GGA      1536
Met Pro Ser Leu His Asn Ile Pro Val Pro Glu Thr Asn Tyr Leu Gly
            500                 505                 510
AAT ACA CCC ACC ATG CCA TTC AGC TCC TTG CCA CCA ACA GAT GAA TCT      1584
Asn Thr Pro Thr Met Pro Phe Ser Ser Leu Pro Pro Thr Asp Glu Ser
        515                 520                 525
ATA AAA TAT ACC ATA TAC AAT AGT ACT GGC ATT CAG ATT GGA GCC TAC      1632
Ile Lys Tyr Thr Ile Tyr Asn Ser Thr Gly Ile Gln Ile Gly Ala Tyr
    530                 535                 540
AAT TAT ATG GAG ATT GGT GGG ACG AGT TCA TCA CTA CTA GAC AGC ACA      1680
Asn Tyr Met Glu Ile Gly Gly Thr Ser Ser Ser Leu Leu Asp Ser Thr
545                 550                 555                 560
AAT ACG AAC TTC AAA GAA GAG CCA GCT GCT AAG TAC CAA GCT ATC TTT      1728
Asn Thr Asn Phe Lys Glu Glu Pro Ala Ala Lys Tyr Gln Ala Ile Phe
                565                 570                 575
GAT AAT ACC ACT AGT CTG ACG GAT AAA CAC CTG GAC CCA ATC AGG GAA      1776
Asp Asn Thr Thr Ser Leu Thr Asp Lys His Leu Asp Pro Ile Arg Glu
            580                 585                 590
AAT CTG GGA AAG CAC TGG AAA AAC TGT GCC CGT AAA CTG GGC TTC ACA      1824
Asn Leu Gly Lys His Trp Lys Asn Cys Ala Arg Lys Leu Gly Phe Thr
        595                 600                 605
CAG TCT CAG ATT GAT GAA ATT GAC CAT GAC TAT GAG CGA GAT GGA CTG      1872
Gln Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly Leu
    610                 615                 620
AAA GAA AAG GTT TAC CAG ATG CTC CAA AAG TGG GTG ATG AGG GAA GGC      1920
Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Val Met Arg Glu Gly
625                 630                 635                 640
ATA AAG GGA GCC ACG GTG GGG AAG CTG GCC CAG GCG CTC CAC CAG TGT      1968
Ile Lys Gly Ala Thr Val Gly Lys Leu Ala Gln Ala Leu His Gln Cys
                645                 650                 655
TCC AGG ATC GAC CTT CTG AGC AGC TTG ATT TAC GTC AGC CAG AAC          2013
Ser Arg Ile Asp Leu Leu Ser Ser Leu Ile Tyr Val Ser Gln Asn
            660                 665                 670
TAA                                                                 2016
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 671 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Gln Pro Asp Met Ser Leu Asn Val Ile Lys Met Lys Ser Ser Asp
 1               5                  10                  15
Phe Leu Glu Ser Ala Glu Leu Asp Ser Gly Gly Phe Gly Lys Val Ser
            20                  25                  30
Leu Cys Phe His Arg Thr Gln Gly Leu Met Ile Met Lys Thr Val Tyr
        35                  40                  45
Lys Gly Pro Asn Cys Ile Glu His Asn Glu Ala Leu Leu Glu Glu Ala
    50                  55                  60
Lys Met Met Asn Arg Leu Arg His Ser Arg Val Val Lys Leu Leu Gly
65                  70                  75                  80
Val Ile Ile Glu Glu Gly Lys Tyr Ser Leu Val Met Glu Tyr Met Glu
                85                  90                  95
Lys Gly Asn Leu Met His Val Leu Lys Ala Glu Met Ser Thr Pro Leu
            100                 105                 110
Ser Val Lys Gly Arg Ile Ile Trp Glu Ile Ile Glu Gly Met Cys Tyr
        115                 120                 125
Leu His Gly Lys Gly Val Ile His Lys Asp Leu Lys Pro Glu Asn Ile
    130                 135                 140
Leu Val Asp Asn Asp Phe His Ile Lys Ile Ala Asp Leu Gly Leu Ala
145                 150                 155                 160
Ser Phe Lys Met Trp Ser Lys Leu Asn Asn Glu Glu His Asn Glu Leu
                165                 170                 175
Arg Glu Val Asp Gly Thr Ala Lys Lys Asn Gly Gly Thr Leu Tyr Tyr
            180                 185                 190
Met Ala Pro Glu His Leu Asn Asp Val Asn Ala Lys Pro Thr Glu Lys
        195                 200                 205
Ser Asp Val Tyr Ser Phe Ala Val Val Leu Trp Ala Ile Phe Ala Asn
    210                 215                 220
Lys Glu Pro Tyr Glu Asn Ala Ile Cys Glu Gln Gln Leu Ile Met Cys
225                 230                 235                 240
Ile Lys Ser Gly Asn Arg Pro Asp Val Asp Asp Ile Thr Glu Tyr Cys
                245                 250                 255
Pro Arg Glu Ile Ile Ser Leu Met Lys Leu Cys Trp Glu Ala Asn Pro
            260                 265                 270
Glu Ala Arg Pro Thr Phe Pro Gly Ile Glu Glu Lys Phe Arg Pro Phe
        275                 280                 285
Tyr Leu Ser Gln Leu Glu Glu Ser Val Glu Glu Asp Val Lys Ser Leu
    290                 295                 300
Lys Lys Glu Tyr Ser Asn Glu Asn Ala Val Val Lys Arg Met Gln Ser
305                 310                 315                 320
Leu Gln Leu Asp Cys Val Ala Val Pro Ser Ser Arg Ser Asn Ser Ala
                325                 330                 335
Thr Glu Gln Pro Gly Ser Leu His Ser Ser Gln Gly Leu Gly Met Gly
            340                 345                 350
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Pro Val Glu Glu Ser Trp Phe Ala Pro Ser Leu Glu His Pro Gln Glu
        355                 360                 365
Glu Asn Glu Pro Ser Leu Gln Ser Lys Leu Gln Asp Glu Ala Asn Tyr
    370                 375                 380
His Leu Tyr Gly Ser Arg Met Asp Arg Gln Thr Lys Gln Gln Pro Arg
385                 390                 395                 400
Gln Asn Val Ala Tyr Asn Arg Glu Glu Glu Arg Arg Arg Arg Val Ser
                405                 410                 415
His Asp Pro Phe Ala Gln Gln Arg Pro Tyr Glu Asn Phe Gln Asn Thr
            420                 425                 430
Glu Gly Lys Gly Thr Val Tyr Ser Ser Ala Ala Ser His Gly Asn Ala
        435                 440                 445
Val His Gln Pro Ser Gly Leu Thr Ser Gln Pro Gln Val Leu Tyr Gln
    450                 455                 460
Asn Asn Gly Leu Tyr Ser Ser His Gly Phe Gly Thr Arg Pro Leu Asp
465                 470                 475                 480
Pro Gly Thr Ala Gly Pro Arg Val Trp Tyr Arg Pro Ile Pro Ser His
                485                 490                 495
Met Pro Ser Leu His Asn Ile Pro Val Pro Glu Thr Asn Tyr Leu Gly
            500                 505                 510
Asn Thr Pro Thr Met Pro Phe Ser Ser Leu Pro Pro Thr Asp Glu Ser
        515                 520                 525
Ile Lys Tyr Thr Ile Tyr Asn Ser Thr Gly Ile Gln Ile Gly Ala Tyr
    530                 535                 540
Asn Tyr Met Glu Ile Gly Gly Thr Ser Ser Ser Leu Leu Asp Ser Thr
545                 550                 555                 560
Asn Thr Asn Phe Lys Glu Glu Pro Ala Ala Lys Tyr Gln Ala Ile Phe
                565                 570                 575
Asp Asn Thr Thr Ser Leu Thr Asp Lys His Leu Asp Pro Ile Arg Glu
            580                 585                 590
Asn Leu Gly Lys His Trp Lys Asn Cys Ala Arg Lys Leu Gly Phe Thr
        595                 600                 605
Gln Ser Gln Ile Asp Glu Ile Asp His Asp Tyr Glu Arg Asp Gly Leu
    610                 615                 620
Lys Glu Lys Val Tyr Gln Met Leu Gln Lys Trp Val Met Arg Glu Gly
625                 630                 635                 640
Ile Lys Gly Ala Thr Val Gly Lys Leu Ala Gln Ala Leu His Gln Cys
                645                 650                 655
Ser Arg Ile Asp Leu Leu Ser Ser Leu Ile Tyr Val Ser Gln Asn
            660                 665                 670
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WHAT IS CLAIMED IS:

1. An isolated, recombinant nucleic acid encoding a human Receptor Interacting Protein (hRIP) kinase domain.

2. An isolated, recombinant nucleic acid encoding a human Receptor Interacting Protein (hRIP) comprising SEQ ID NO: 1.

3. A method of making a human Receptor Interacting Protein (hRIP) kinase domain containing protein, said method comprising the steps of translating a nucleic acid according to claim 1 to form a translation product and isolating said translation product.

4. A method of identifying lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease, said method comprising the steps of:

making a protein according to the method of claim 3,

forming a mixture comprising:

said protein,

a natural intracellular hRIP binding target, wherein said binding target is capable of specifically binding said protein, and

a candidate pharmacological agent;

incubating said mixture under conditions whereby, but for the presence of said candidate pharmacological agent, said protein selectively binds said binding target at a first binding affinity;

detecting a second binding affinity of said protein to said binding target,

wherein a difference between said first and second binding affinity indicates that said candidate pharmacological agent is a lead compound for a pharmacological agent capable of modulating hRIP-dependent signal transduction.

5. A method according to claim 4, wherein said hRIP binding target comprises a Tumor necrosis factor receptor Associated Factor -2 (TRAF2) or a Tumor necrosis factor Receptor-1 Associated Death Domain protein (TRADD).

6. A method of identifying lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease, said method comprising the steps of:

making a protein according to the method of claim 3,

forming a mixture comprising:

said protein,

an hRIP substrate, wherein said hRIP kinase domain of said protein is capable of specifically phosphorylating said substrate, and

a candidate pharmacological agent;

incubating said mixture under conditions whereby, but for the presence of said candidate pharmacological agent, said hRIP kinase domain selectively phosphorylates said substrate at a first rate;

detecting a second rate of phosphorylation of said substrate by said hRIP kinase domain,

wherein a difference between said first and second rate indicates that said candidate pharmacological agent is a lead compound for a pharmacological agent capable of modulating hRIP kinase activity.

7. A method according to claim 6 wherein said hRIP substrate is hRIP.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16778

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL : 536/23.5; 435/ 69.1, 69.5, 252.3, 320.1; 530/350, 351
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/ 69.1, 69.5, 252.3, 320.1; 530/350, 351

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>---<br>Y | STANGER et al. RIP: A Novel Protein Containing a Death Domain That Interacts with Fas/APO-1 (CD95) in Yeast and Causes Cell Death. Cell. 19 May 1995, Vol. 81, pages 513-523, see Figs. 2-3, and sequence alignment, | 1-3<br>------<br>2 |
| Y, P | WO 96/25941 A1 (YEDA RESEARCH & DEVELOPMENT CO. LTD.) 29 August 1996 (29/08/96), see abstract , figures and claims. | 1-3 |
| A | HSU et al. The TNF Receptor 1-Associated Protein TRADD Signals Cell Death and NF-kB Activation. Cell, 19 May 1995, Vol. 81, pages 495-504, see all. | 1-3 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 JANUARY 1997 | 28 FEB 1997 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. (703) 305-3230 | Authorized officer<br>GARNETTE D. DRAPER<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16778

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A, P | BAKER et al. Transducers of Life and Death: TNF Receptor Superfamily and Associated Proteins. Oncogene, 04 January 1996, Vol. 12, pages 1-9, see all | 1-3 |

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16778

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 f first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-3

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/16778

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 21/04; C12P 21/06, 21/02; C12N 1/20, 15/00; C07K 1/00, 14/52

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-3, drawn to nucleic acids that encode for human Receptor Interacting Proteins (hRIP) and methods of making the encoded proteins.

Group II, claims 4-7, drawn to methods of identifying lead compounds.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of Group I is directed to nucleic acids that encode for hRIP and to methods of making hRIP; whereas the special technical feature of Group II is directed to methods of identifying lead compounds. The methods of these two groups do not share a special technical and unifying feature, because each of these methods require the utilization of different process/method steps, different elements/agents, and their are different starting material and the final outcomes are also different. Furthermore, these methods and their steps and elements are not required one for the other.